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ISOLATION AND CRYOGENIC PRESERVATION OF MONOCYTES FROM
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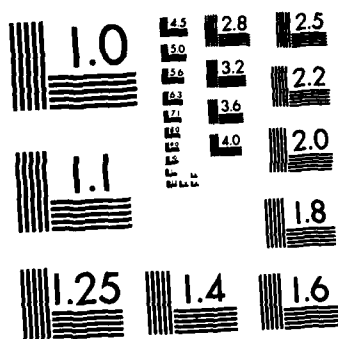
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ISOLATION AND CRYOGENIC PRESERVATION OF
MONOCYTES FROM PLATELETPHERESIS CELLULAR
RESIDUES

Prepared for publication in TRANSFUSION

Center for Blood Research
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Abstract (continued)

nine percent of isolated monocytes were viable in the fluorescein diacetate (FDA)-ethidium bromide (EB) test. Myeloperoxidase-positive cells were 95% and 90% respectively in Chambers #1 and #2. Ninety-four percent of monocytes ingested 5 or more opsonized Fluolite particles and 95% ingested 1 or more ethidium-treated zymosan particles.

After storage in liquid nitrogen for up to 9 weeks, 99% of the cells were recovered after thawing. Of these, 95% were myeloperoxidase-positive, 94% showed intact membranes in the FDA-EB test, 95% ingested 5 or more opsonized Fluolite particles, and 96% ingested 1 or more ethidium-treated xymosan particles. ←

The results demonstrate the utility of elutriation as a means to isolate large numbers of monocytes. The isolated cells can be cryogenically preserved for at least 2 months with small loss of phagocytic function.

ABSTRACT

Human monocytes were isolated from the cellular residues after plateletpheresis of donors using the Haemonetics Model 30 Blood Cell Processor. Mononuclear cells were obtained with Ficoll-Isopaque and separated from lymphocytes by stepwise elutriation in a Beckman JE-6 rotor equipped with two isolation chambers. The isolated cells were frozen in a solution containing an extracellular (hydroxyethyl starch, HES) and an intracellular (dimethylsulfoxide, DMSO) cryoprotective compound.

In three procedures, approximately 1×10^9 monocytes were obtained. Ninety-nine percent of isolated monocytes were viable in the fluorescein diacetate (FDA)-ethidium bromide (EB) test. Myeloperoxidase-positive cells were 95% and 90% respectively in Chambers #1 and #2. Ninety-four percent of monocytes ingested 5 or more opsonized Fluolite particles and 95% ingested 1 or more ethidium-treated zymosan particles.

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The results demonstrate the utility of elutriation as a means to isolate large numbers of monocytes. The isolated cells can be cryogenically preserved for at least 2 months with small loss of phagocytic function.

Keywords: Monocytes; Isolation; Cryopreservation; Plateletpheresis; Cellular Residues from Counterflow Centrifugation; Recovery > 90%; Function > 90%

INTRODUCTION

Elutriation in a counterflow rotor has been shown to be an efficient method for isolating monocytes from blood or buffy coat white cells (1,2). Mononuclear cells were exposed at moderate centrifugal forces and high dilutions in a Beckman JE-6 rotor to counterflow of buffer solutions under conditions in which sedimentation rate was proportional to cell diameter (3). Thus, lymphocytes and monocytes of similar densities obtained in an isopycnic centrifugation, were separated according to size (1). The monocytes obtained exhibited normal morphology and phagocytosis and survived freezing and storage in liquid nitrogen for 1 month.

During plateletpheresis, large numbers of red cells and mononuclear cells are removed by centrifugation. The cellular residues after removal of platelets may contain between 1×10^9 and 1×10^{10} monocytes. We have subjected these residual cells to centrifugation with Ficoll-Isopaque and the mononuclear fraction derived to counterflow centrifugation. The principles and significant data involved in isolation and preservation of monocytes from whole blood have been published, including preliminary data obtained with monocytes obtained as by-product cells of plateletpheresis (1). This paper extends these findings and amplifies the isolation and cryogenic preservation of monocytes from platelet bags wherein 1×10^9 monocytes of high purity may be routinely collected in suspension. These may be frozen with the same protocol previously developed for granulocytes (2).

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MATERIALS AND METHODS

Monocytes from Plateletpheresis Cellular Residues

Plateletpheresis was performed at the American Red Cross Blood Services Center, Boston, MA. Whole blood was passed into a 375 ml disposable plastic bowl in a Haemonetics 30 Blood Cell Processor. The platelet-rich band and the buffy coat were aspirated into a bag for platelet concentrates, and the packed cells in plasma were returned to the donor. This cycle was carried out 7-8 times. The pooled platelet-rich concentrate was centrifuged at $150 \times g$ for 9 minutes in order to separate platelets and plasma from unwanted red blood cells and leukocytes. The 10-15 ml of residual pelleted blood cells (plateletpheresis residue) was the starting material for monocyte separations.

Isolation of Monocytes by Centrifugal Cytography in the Counterflow Centrifuge

Ten to 12 ml of the residual cells were diluted 3-fold with a solution made with equal volumes of Type AB plasma and phosphate-buffered saline (PBS, pH 7.1). Fifteen ml of the diluted suspension of cells were layered on 15 ml of Lymphoprep (Ficoll-Isopaque, Nyegaard and Co., Oslo, Norway) in a 50 ml Corning polypropylene screw-top tube and the buoyant mononuclear fraction removed after centrifugation at $400 \times g$ for 40 minutes at room temperature. The mononuclear cell suspension was removed and diluted 2-fold with PBS (318 mOsm/kg H₂O) containing 3 mM EDTA and 1.2% (w/v) human serum albumin. The diluted suspension was incubated for 15-

20 minutes. After centrifugation at 400 X g for 15 minutes the supernatant containing platelets was aspirated and the mononuclear cell pellet resuspended in 20-25 ml of the hypertonic PBS buffer. The mononuclear population was then subjected to counterflow centrifugation in the system previously described (3). The diluted plateletpheresis residue cells were injected into the system through a 3-way stopcock, and the cells mixed with the buffer in the stoppered mixing tube. Monocytes were held in the two separation chambers within the rotor while platelets, red cells, and lymphocytes were flushed into an external receptacle. Flow rates, centrifugal forces, and other conditions were similar to those previously described (1,3). These were 8.2 ml/minute initially and increased to 9.4 ml/minute over a 60-minute interval. White cell differential counts made with Wrights-stained smears, myeloperoxidase, estimates of viability with FDA and EB, tests of phagocytosis with EB-treated zymosan and opsonized Fluolite, and volume plots made with a Coulter Counter ZH and Channelyzer were done as previously described (1,4). From each of the two separation chambers, 4 ml of monocyte suspension was aspirated into a 17 mm X 100 mm polypropylene tube in an ice bath and the suspensions diluted with an equal volume of cold PBS (0.15 M NaCl, 0.01 M phosphate, 1.2% human serum albumin and 3 mM EDTA).

Preservation of Monocytes

Monocytes obtained in this manner were frozen with the same cryogenic procedure and conditions as previously described (1,2,5). One significant

difference was that the numbers of monocytes in the suspensions were 10 times greater than those obtained from whole blood. One volume of cryoprotective solution at 4 C was added dropwise with mixing to one volume (approximately 8 ml) of monocytes from each chamber. The final concentration of cryoprotectants, when mixed, was 5% DMSO, 6% HES (McGaw, HMW 150,000), 4% HSA (human serum albumin) and 56 mM glucose in Normosol-R (Abbott), pH 7.1. The mixture was divided into 2 ml aliquots containing 60×10^6 - 70×10^6 monocytes. They were placed in 17 mm X 100 mm polypropylene tubes on ice for 5-10 minutes, cooled at 4 C per minute to -80 C, and stored in liquid nitrogen (-197 C). The tubes were frozen for up to 71 days. Monocytes prepared as above, but not frozen, served as controls. Monocytes were removed from the liquid nitrogen freezer, immediately placed in a 42 C water bath and shaken manually until all ice dissolved except for a small pellet (usually within 4 minutes). The tube was removed from the bath and thawing continued at room temperature with swirling until the temperature reached approximately 10 C. The suspension was diluted to reduce the DMSO concentration for testing by adding, dropwise over 3-5 minutes, 3 volumes of a solution at room temperature, composed of 7% HES, 4% HSA, and 28 mM glucose in Normosol-R, pH 7.1. The suspensions were maintained at 2 C on ice for testing and subsequent washing.

RESULTS

The recoveries of monocytes in three detailed studies are reported in Table 1. The total leukocytes in the residues ranged from 2.0×10^9 to 7.5×10^9 containing 0.8×10^9 to 1.6×10^9 monocytes. Of $4.3 \pm 1.7 \times 10^9$ average leukocytes available, 94% were mononuclear (monocytes and lymphocytes) and 5% were polymorphonuclear granulocytes. Therefore, on the basis of white cell differential count and percentage of myeloperoxidase-positive cells, the mononuclear population contained $1.1 \pm 0.5 \times 10^9$ monocytes. Membrane integrity values as high as $97 \pm 2\%$ were FDA-positive and only $3 \pm 2\%$ reacted with EB. After gradient centrifugation on Ficoll-Isopaque, 84% of the monocytes were recovered in the buoyant mononuclear layer. These exhibited no loss of membrane integrity, as determined by the FDA test, or myeloperoxidase activity. Following counterflow elutriation, 0.6×10^9 cells were recovered in Chamber #1 ($64 \pm 1\%$). These cells were 99% mononuclear and 95% myeloperoxidase-positive, indicating approximately 5% lymphocytes. The remainder of available monocytes, $0.4 \pm 0.1 \times 10^9$, was isolated in Chamber #2. These were $96 \pm 5\%$ mononuclear and $90 \pm 1\%$ myeloperoxidase-positive, indicating 10% of the cells in Chamber #2 were non-phagocytic (lymphocytes).

TABLE 1

Monocytes preserved by freezing yielded high recoveries after thawing (Table 2). Data are given only for cells from Chamber #1. Similar results were obtained with the cells preserved from Chamber #2 (not shown). The recoveries at each stage showed small losses, the major ones occurring after thawing and dilution to reduce DMSO, and small additional losses after incubation with particles and washing.

TABLE 2

The volume distributions of preserved monocytes showed two white cell peaks, monocytes at median channel numbers 51 to 53 and contaminating lymphocytes between 29 and 34. Monocytes and lymphocytes as isolated, frozen-thawed and examined, exhibited little volume change during preservation, thawing and washing. Monocytes from Chamber #2 were similar (not shown).

The function of preserved monocytes was well maintained as reflected in myeloperoxidase-positive cells, ethidium viability and phagocytic indices. Comparison of frozen, thawed, diluted and washed monocytes with unfrozen control cells showed comparable values of myeloperoxidase activity and comparable phagocytic indices in high, medium, low and zero categories for opsonized Fluolite (Table 3). However, a significant difference in Fluolite and zymosan ingestion between fresh and frozen cells was observed in the high particles per cell category. The percent of monocytes ingesting greater than 15 Fluolite particles and greater than 5 zymosan particles was substantially reduced after freezing and thawing, showing a shift in pre-frozen levels from a mean of 18% to 6% for Fluolite and from 44% to 26% for zymosan.

TABLE 3

On two separate occasions monocytes preserved for two months in liquid nitrogen, thawed, washed, and cultured for ten days were found to differentiate morphologically into macrophages (not shown).

DISCUSSION

A limiting factor in monocyte experimentation has been technical difficulties in obtaining relatively large numbers of monocytes in suspension. These have been obtained by gradient centrifugation of buffy coat cells or whole blood in bovine serum albumin (6), sucrose (7), or Ficoll-Isopaque (8). Monocytes were isolated from mixtures of lymphocytes and monocytes by adherence to glass (9) or plastic surfaces (10). Monocytes isolated by gradient centrifugation are usually few in number and of variable purity. Adherence methods produce more pure cell populations but function and yield are affected by the detachment procedure. Ackerman and Douglas (11) obtained a relatively pure (92%) and functional (95% phagocytic) monocyte suspension by using EDTA to reverse the adhesion of monocytes from heparinized whole blood (approximately 1×10^7 cells) to micro exudate-coated plastic surfaces. Counterflow centrifugal elutriation was described by Sanderson et al (12) as a method for obtaining monocytes in suspension. Utilizing a single isolation chamber in a counterflow rotor, this procedure produced 1.5×10^6 to 3.0×10^6 suspended monocytes. The isolated cells from 10 ml of whole blood contained 10% lymphocytes and the cells were 99% functional. Loos et al (13) utilized a discontinuous Ficoll-Isopaque gradient to obtain monocytes in suspension. By this method 5×10^6 monocytes were obtained from 50 ml of whole blood and 50×10^6 monocytes from full units of whole blood with 90-92% purity.

Starting with residual cells in platelet bags collected by pheresis,

use of dual chambers in the Beckman JE-6 rotor enabled us to isolate 1.0×10^9 monocytes of 95% purity. The number of monocytes was 20 times that isolated previously from 100 ml of whole blood, and 200 to 400 times that isolated from 10 ml of whole blood by Sanderson et al (12). Monocytes isolated this way were found to have high values for cytoplasmic and nuclear membrane integrity, myeloperoxidase activity, and phagocytic indices.

Preservation was accomplished by using the 5% DMSO and 6% HES method we developed for cryopreserving granulocytes (1,2,5). These monocytes, isolated from HLA-typed donors, survived freezing and storage in liquid nitrogen for periods up to 71 days with minimal loss of function. The availability of large numbers of phagocytic white cells stable to long-term preservation enhances the prospects for transfusion of HLA-matched monocytes. Preservation effects on immune functions and surface markers of monocytes are important considerations currently under investigation.

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TABLE 1

ISOLATION OF HUMAN MONOCYTES FROM PLATELETPHERESIS RESIDUES

Sample n = 3	Total Leukocytes x 10 ⁹	Myelo- peroxidase- Positive (%)	Differential Counts			Total Monocytes x 10 ⁹	Recovery from Buffy Coat (%)	Viable Cells	
			PMN	Mononuclear	Other			FDA (%)	EB (%) Median Channel #
Diluted Residue	4.3 (2.4-5.3)	23 (18-32)	5 (3-6)	94 (93-97)	1 (0-1)	1.1 (0.8-1.6)		97 (95-98)	3 (2-5) (65-67)
Ficoll- Isopaque Washed	3.5 (2.5-4.6)	31 (28-32)	1	99	0	1.0 (0.8-1.3)	84* (82-86) 86** (82-90)	98 (97-99)	2 (1-3) (62-68)
Elutriated Chamber #1	0.6 (0.6-0.7)	95 (90-94)	0	99 (99-100)	1 (0-1)	0.6 (0.5-0.6)	64 (63-66) 66 (64-67)	99 (97-100)	1 (0-3) (59-64)
Elutriated Chamber #2	0.4 (0.4-0.5)	90 (90-91)	1 (0-1)	96 (91-99)	4 (1-8)	0.4 (0.2-0.5)	36 (31-38) 32 (30-37)	97 (96-97)	3 (3-4) (63-67)

FDA and EB are the percentage of monocytes fluorescing with fluorescein diacetate and ethidium bromide.

*Recovery calculated from counts from the Coulter Counter Model F.

**Recovery calculated from the number of monocytes in the populations determined with the Coulter Model ZH and Channellyzer. The numbers are averages. The range is given in parenthesis.

TABLE 2
CRYOPRESERVATION OF MONOCYTES

<u>Sample</u>	<u>Recovery (%)</u>	<u>Median Channel</u>
Prefrozen n = 3	from residue: 84* (83-86) 86 (84-87)	54 (53-57)
Thawed n = 9	from mononuclear cells: 100 99	53 (50-58)
Thawed and Diluted n = 9	94 (92-100) 97 (94-100)	53 (49-57)
Thawed, Diluted and Incubated** n = 9	95 (92-100) 95 (81-100)	54 (52-57)
Control n = 3	-- --	62 (59-64)

The mean and range (parenthesis) of each value is reported.

*Recoveries were calculated as in Table 1

**Thawed, diluted cells were incubated in 37 C water bath for 20 minutes

TABLE 3

FUNCTION OF CRYOPRESERVED MONOCYTES

Sample	Myeloperoxidase Positive (%)	Viable Cells FDA EB	Fluolite			Phagocytosis			Zymosan
			>15	6-15 Particles	% of Cells Containing 0-5	>5	1-5 Particles	0	
Prefrozen n = 3	94 (90-97)	98 (96-99)							
Thawed* n = 9	93 (86-96)	97 (93-98)							
Thawed and Diluted n = 9	95 (89-97)	96 (94-99)							
Thawed, Diluted, Incubated n = 9	95 (89-98)	94 (92-98)	6 (0-12)	53 (30-68)	37 (18-60)	26 (17-37)	71 (30-78)	4 (1-5)	
Control n = 3	95 (90-94)	99 (97-100)	18 (9-22)	56 (48-60)	21 (14-34)	44 (17-57)	51 (37-80)	5 (3-6)	

*For samples with n = 9, values are averages \pm S.D.

** Phagocytosis was not studied in the prefrozen, thawed, and thawed and diluted suspensions as previous studies (2) with granulocytes had shown myeloperoxidase activity was inhibited by 5% DMSO, and particle ingestion was very low at 20 - 40C.

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